

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  C12N 15/00, 15/85, A01K 67/00	A1	(11) International Publication Number: WO 90/11355  (43) International Publication Date: 4 October 1990 (04.10.90)
(21) International Application Number: PCT/GB90/00388		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.
(22) International Filing Date: 15 March 1990 (15.03.90)		
(30) Priority data: 8906214.5 17 March 1989 (17.03.89) GB		Published <i>With international search report.</i>
(71) Applicant ( <i>for all designated States except US</i> ): NATIONAL RESEARCH DEVELOPMENT CORPORATION [GB/GB]; 101 Newington Causeway, London SE1 6BU (GB).		
(72) Inventor; and		
(75) Inventor/Applicant ( <i>for US only</i> ) : SIMKISS, Kenneth [GB/GB]; Foxhill, Foxhill Lane, Playhatch, Reading, Berkshire RG4 9QF (GB).		
(74) Agent: PERCY, Richard, Keith; Patent Department, National Research Development Corporation, 101 Newington Causeway, London SE1 6BU (GB).		

(54) Title: INTRODUCING AN EXOGENOUS GENE INTO BIRDS

(57) Abstract

A method of introducing foreign nucleic acid, especially a gene into birds, especially chickens, takes advantage of the developmental stage of the embryo into which primordial germ cells (PGCs) collect in the germinal crescent, migrate in the blood stream and settle in the germinal ridge, which becomes the gonad. The method comprises providing *in vitro* foreign nucleic acid which it is desired to introduce into the germ line of a bird, introducing the foreign nucleic acid into explanted primordial germ cells of a bird and then introducing these primordial germ cells into the blood system of a recipient embryo of an incubated egg, at a stage of embryonic development at which introduced primordial germ cells will settle in the germinal ridge.

BEST AVAILABLE COPY

## **DESIGNATIONS OF "DE"**

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

### ***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT Austria	ES Spain	MG Madagascar
AU Australia	FI Finland	ML Mali
BB Barbados	FR France	MR Mauritania
BE Belgium	GA Gabon	MW Malawi
BF Burkina Faso	GB United Kingdom	NL Netherlands
BG Bulgaria	HU Hungary	NO Norway
BJ Benin	IT Italy	RO Romania
BR Brazil	JP Japan	SD Sudan
CA Canada	KP Democratic People's Republic of Korea	SE Sweden
CF Central African Republic	KR Republic of Korea	SN Senegal
CG Congo	LJ Liechtenstein	SU Soviet Union
CH Switzerland	LK Sri Lanka	TD Chad
CM Cameroon	LU Luxembourg	TG Togo
DE Germany, Federal Republic of	MC Monaco	US United States of America
DK Denmark		

- 1 -

### INTRODUCING AN EXOGENOUS GENE INTO BIRDS

#### Background of the invention

##### 1. Field of the invention

This invention is in the field of transgenic animals.

##### 2. Description of the prior art

05 There are basically three ways to introduce exogenous (foreign) genes into embryos. These are by direct injection into the pronucleus, by infection of early embryos with genetically manipulated viruses, or by the transfer of cells with a modified genotype into the blastocyst to produce a chimera. The value of  
10 each of these approaches varies with the nature of the embryological system involved and in this context birds have been particularly difficult to use.

Injection of genes into pronuclei has been successful in mice, rabbits, sheep etc. but attempts to produce transgenic  
15 birds by direct injection of DNA into the pronucleus have been largely unsuccessful because it is difficult to locate the pronucleus within the body of a large yolk egg, H. Sang & M.M. Perry, Molecular Reproduction & Development 1, 98-106 (1989). Infection of eggs with retroviruses is not a reliable method.  
20 Thus, it has been reported by R.A. Bosselman *et al.* (of Amgen Inc.), Science 243, 533-535 (27 January 1989) that only 8% infection of the germ cell line occurred.

#### Summary of the invention

It has now been found that foreign nucleic acid, especially  
25 foreign gene(s), can be introduced into primordial germ cells derived from the embryo of a donor bird, that the cells carrying the foreign nucleic acid can be introduced into the embryo of a recipient bird, and that the foreign nucleic acid is thereby carried into the germ cells of the embryo and therefore into the  
30 germ line of a bird which will be produced from the embryo. In this way, transgenic birds, especially poultry and game birds, can be produced.

As long ago as 1965, A.W. Blackler, J. Embryol. Exp. Morphology 13, 51-61 (1965), showed that primordial germ cells

- 2 -

could be grafted at the neurula stage from one embryo to another in toads (Xenopus laevis) and demonstrated the transfer by reference to sex control of the resulting tadpoles.

Attempts have been made previously to transfer primordial  
05 germ cells between species or from donor embryos of birds to sterilized recipients. It has, however, been difficult to establish unequivocally whether these cells survived the attempted transfer and to guarantee the efficiency of the sterilization process, see G. Reynaud, Wilhelm Roux Archiv für  
10 Entwicklungsmechanik der Organismen (Roux's Archives of Developmental Biology) 179, 85-110 (1976). For these reasons, transfer of primordial germ cells has not previously come seriously into reckoning as a practical way of producing transgenic birds. In the present invention, however, the  
15 inventor has found that transfer can be achieved and be demonstrated to have been achieved, and moreover in a significant number of experiments, and in so doing has opened up a new and practical route for the production of transgenic birds. Further, he has found that it is possible to transfer foreign nucleic acid,  
20 via explanted primordial germ cells, into the germ cell line of an embryo.

The invention herein can be expressed as a method of introducing foreign nucleic acid, especially a foreign gene, into birds, which comprises providing in vitro foreign nucleic acid  
25 which it is desired to introduce into the germ line of a bird, introducing the foreign nucleic acid into explanted primordial germ cells of a bird and then introducing these primordial germ cells into the blood system of a recipient embryo of an incubated egg, at a stage of embryonic development at which introduced  
30 primordial germ cells will settle in the germinal ridge.

Brief description of the drawings

Figure 1 shows a chicken embryo in surface view;

Figure 2 is a graph of concentration of primordial germ cells in blood plotted against time (stage of embryonic development);

35 Figure 3 is a transverse section of part of a chick embryo

- 3 -

showing the germinal ridge; and

Figures 4 and 5 show proviral DNA constructs used to prepare a replication-defective retroviral vector carrying a foreign gene, this vector being used to infect donor primordial germ cells.

Description of the preferred embodiments

Primordial germ cells (PGCs) are the cells which are destined to give rise to gametes (eggs and spermatazoa). They are large cells (larger than blood cells, for example), typically of diameter up to 20 micrometres. After an egg has incubated for about 4 hours, PGCs can be seen in the germinal crescent which lies outside the embryo (Figure 1). In Figure 1, the donor embryo 1 has primordial germ cells 2 in the germinal crescent 3. The germinal crescent lies between the area opaca and area pellucida anterior to the head of the developing embryo. Also shown are blood vessels 4 and the gonad 5 of the donor embryo. The PGCs migrate from this site of origin, via the bloodstream, to the site of the future gonad, which is called the germinal ridge. In the embryo of the domestic fowl, this migration occurs after about 50h of incubation at stage 16 of development (Figure 2: "A"). It is seen as a large pulse of transient primordial germ cells among the normal erythrocytes of the blood. Within a few hours they disappear from the blood and settle in the germinal ridge (Figure 2: "B"). Figure 2 shows the population of PGCs in the blood stream of the embryo plotted against somite number, which represents a stage of embryonic development. (The somite is a block of muscle. By counting these somites in the head to tail direction of the embryo, its development can be quantised). Figure 3 is a transverse section of a part of the embryo showing the germinal ridge. 11 = mesoderm, 12 = spinal cord, 13 = extra-embryonic cavity, 14 = blood vessel, 15 = PGCs carried in the bloodstream, 16 = germinal ridge.

Once settled in the germinal ridge, the PGCs proliferate to form germ cells. The number settling is of the order of a few hundred, while the number of proliferated germ cells produced is

- 4 -

of the order of a million in the female. Only a fraction of these germ cells (a few thousand) are carried through to the adult bird, there being extensive atrophy of these oocytes after hatching.

05 Virtually all domestic fowl contain DNA copies of retroviral genomes that have entered the germ line during evolution but are inherited as structural genes. In this invention such individuals have been used as donors to inject primordial germ cells into a "line zero" strain of retrovirus-free birds. Blood,  
10 containing PGCs, was taken from an embryo of a donor bird and injected into the heart of a recipient embryo. After further incubation, the recipient embryos were dissected and DNA extracted from the gonads, heart, liver and muscle. Untreated donor and recipient embryos were also dissected to provide  
15 positive and negative controls. Dot blots and Southern blots were prepared and probed with labelled retroviral DNA. In 4 out of 11 transfer experiments the gonads were found to be labelled with donor cell DNA. In one case the heart was also positive but in other cases the transfer was specific for the gonad tissues.

20 The results indicate that it is possible to transfer primordial germ cells between individuals and obtain the clonal growth of these stem cells in recipient embryos. It is known that some primordial germ cells do not enter the germinal ridge but settle in other organs of which the heart is most common.  
25 Inevitably some nucleated red blood cells were also transferred in these experiments but these appear to have been diluted out during embryo growth so that in most transfers there is no donor DNA in other tissues.

Very little is known of the fundamental processes involved in  
30 the formation of the avian gonad. It may be assumed that half the transfers involved in these experiments were trans-sexual (i.e. male primordial germ cells into female birds and vice versa). It is also to be expected that there would be competition between donor and recipient primordial germ cells.  
35 Despite this, there is a high success rate in affecting transfers

- 5 -

so that this is a potentially effective way of producing birds with chimaeric gonads.

In the present invention it is preferred to take the PGCs from the blood stream of the donor embryo, or from a germinal crescent, in order most closely to simulate the natural processes. There appears to be some chemical signal which causes the germ cells to settle in the germinal ridge and it is not certain whether very immature PGCs would have the means of recognising or following the signal.

The PGCs will normally be explanted, and possibly then cultured in vitro. In order to prevent them from maturing and differentiating too rapidly, before the foreign nucleic acid can be introduced and the PGCs inoculated into the recipient embryo, it will probably be advisable to introduce Differentiation 15 Inhibitory Activity factor into the culture : see Example 1 for references.

The nucleic acid can then be introduced into the PGCs in vitro in any of the now well accepted ways for introducing nucleic acid into cells, e.g. by calcium phosphate transfection 20 of DNA, direct inoculation of DNA into the PGCs or infection of the culture with a retroviral vector carrying nucleic acid foreign to the bird. In all these instances, the usual techniques apply. Thus the direct inoculation technique is similar to that used for inoculating a pronucleus in conventional 25 transgenic technology. Preferably a retroviral vector is used. The vector is preferably one which is not capable of undergoing replication in the PGCs and will normally be formed from two or more DNA constructs which, when acting together, allow integration of the foreign nucleic acid into the germ cell 30 chromosomes.

While normally the explanted PGCs will be isolated from surrounding tissue, it is also possible to introduce the foreign nucleic acid into a sample of PGC-containing tissue, such as blood of the donor embryo (at a stage of development at which 35 PGCs will be present). In particular the blood can be infected

- 6 -

with an appropriate retroviral vector carrying the foreign nucleic acid.

The nucleic acid transferred can be RNA or DNA; DNA can be genomic or cDNA produced from genomic or mRNA. It can be a  
05 polynucleotide coding for a polypeptide. In all cases, appropriate promoters, signal and transcription-termination sequences may be required, as is recognised in the art.

The gene introduced may be any of those familiar to those skilled in the poultry genetics field, including growth genes,  
10 genes which may impart resistance to poultry diseases such as coccidiosis, Marek's Disease Virus, Newcastle Disease Virus, Infectious Bronchitis Virus, Infectious Bursal Disease Virus and so forth. Further, genes coding for attenuated strains of Salmonella may be of value.

15 It is desirable that the competition between native PGCs of the recipient embryo and the donor PGCs containing the foreign nucleic acid for lodgement in the germinal ridge, should be altered in favour of the donor PGCs. One method of achieving this is simply to reduce the ratio of native to introduced PGCs.  
20 Thus, the germinal crescent PGCs of the recipient embryo may be removed by cauterisation or by selective irradiation or by use of a chemosterilant such as the drug Busulphan. Alternatively, the donor PGCs could be cultured and introduced in such large numbers as to swamp the native PGCs.

25 The invention includes all conventional further steps downstream of the introduction of the foreign nucleic acid to the recipient embryo, such as hatching chicks, rearing birds from these chicks, using the eggs of reared egg-laying birds as a source of genes for a further gene transfer and so on. The term  
30 "chick" herein denotes the newly hatched offspring of any bird, not necessarily of a fowl unless the context so requires.

The invention further includes "indirect products of the method" viz. birds which are made transgenic or carry foreign nucleic acid in their germ line through application of the  
35 process of the invention to their mother or to any maternal

- 7 -

ancestor of the bird's mother or father.

The invention is of significance chiefly for gene transfer using cells of the same species or even strain and preferably in poultry such as chickens, ducks, turkeys, geese and guinea fowl  
05 or game birds such as pheasants, grouse and partridges.

The following Examples illustrate the invention. All stages of embryonic development are on the standard Hamburger - Hamilton scale.

EXAMPLE

10 This Example demonstrates in section A that retrovirally infected PGCs can be transferred to retrovirus-free eggs, producing a retrovirally infected embryo. Section B indicates how advantage is taken of this finding to introduce a foreign gene into differentiated tissue of the embryo.

15 A. Transfer of Primordial Germ Cells (PGCs)

1. A normal strain of Rhode Island Red hens was used as the "donor". "Donor" eggs from Rhode Island Red hens as above were incubated to developmental stage 16 and recipient eggs to roughly stage 14-15.
- 20 2. A needle and tube were prepared to collect the blood. This consists of a 30 gauge dental needle attached to a 30 cm length of canula tubing ending in a mouth piece. The needle was dipped into heparin (anti-coagulant) solution before use.
3. A donor egg was positioned beneath a binocular microscope with fibre optic illumination and the embryo exposed by removing the shell over the blunt end (air space) of the egg.
- 25 4. The needle was inserted into a large vein in the donor embryo, usually the one running down the back of the embryo. Alternatively any other vein or the heart may be used. About 10 $\mu$ l of blood, typically containing about 40 PGCs, were drawn into the needle by gentle suction. Alternatively a small syringe may be used to suck the blood into the tube.
- 30 5. The "recipient" embryo was derived from a retrovirus-free White Leghorn strain. Such a strain is kept at the Institute for Animal Health, Houghton Laboratory, Houghton, Huntingdon PE17
- 35

- 8 -

2DA, England. Eggs from this strain are freely available for sale (and have been sold). An alternative source of supply of retrovirus-free eggs is the USDA station at East Lansing, Michigan, USA. The "recipient" egg was laid on its side and its  
05 air space pierced with a pair of fine forceps. The egg was wiped with 70% alcohol and a small hole ( $1\text{ cm}^2$ ) cut in the centre of the shell with a sterile hacksaw blade. The shell and shell membranes were removed with fine forceps. At this stage, the embryo drops away from the shell as the contents displace the air  
10 from the air space.

6. The recipient embryo was located and the blood injected directly into the heart or into the region around the heart or a major blood vessel.

7. The hole in the eggshell was sealed with adhesive tape.

15 8. The egg was incubated in a normal incubator and candled regularly to check growth.

9. After a total of 16/17 days of incubation, which is the time at which the avian embryo develops the maximum number of oocytes, the recipient embryos were dissected and DNA was extracted from  
20 the gonads, heart, liver and muscle, by the method of J.B. Dodgson et al., Cell 17, 879-887 (1979). The DNA was dot-blotted or Southern blotted onto nitrocellulose paper.

10. The blots were probed with radiolabelled retroviral DNA. The retroviral DNA actually used was from a plasmid designated pRAV-1  
25 used at the Institute for Animal Health, Houghton (see above), but originally a gift from Paula Enrietta of the Imperial Cancer Research Foundation, London. It is available from the Institute, but it is not necessary to use this specific probe. Any of the well known avian retroviral DNA clones will do. (To check that a  
30 particular retroviral DNA is suitable is a simple matter, as it lights up certain distinctive bands when DNA taken from avian red blood cells is Southern-blotted and probed with radiolabelled retroviral DNA). The nitrocellulose filters were hybridised overnight and washed at high stringency ( $65^\circ\text{C}$ , 0.1% SSC). In 4  
35 out of 11 experiments the retroviral DNA was found in the gonad

- 9 -

tissue.

B. Gene Manipulation of Primordial Germ Cells

1. It is the intention to take primordial germ cells from the blood sample obtained in processes A1-A4 or alternatively to 05 isolate them from the germinal crescent and maintain the cells in normal avian tissue culture solution (e.g. Eagles modified medium with foetal calf serum).
2. Where necessary, the explanted primordial germ cells would be maintained in an undifferentiated state using developmental 10 inhibitory activity factor, R.L. Williams *et al.*, Nature 336, 684 - 687 (1988), A.G. Smith *et al.*, Nature 1988, 336, 688-685 (1988)
3. Gene insertion into the cells would be by conventional methods (Calcium phosphate incorporation; DNA injection or defective retroviral infection).
- 15 4. Manipulated primordial germ cells would be inserted into embryos as in steps A5-A8 except that these embryos would have had their germinal crescent cells destroyed (by cautery, irradiation or chemosterilization) or, alternatively, excessive donor PGCs would be given before the mobilization of the 20 endogenous PGCs of the recipient embryo.

By these means manipulated primordial germ cells would be introduced into sterilized embryos leading to a permanent modification to the germ line of the bird.

EXAMPLE 2

- 25 This Example demonstrates the infection of PGCs either in vitro or in a donor embryo, with a retroviral vector carrying a foreign gene, transferring the infected PGCs to retrovirus-free eggs and detecting the foreign gene in differentiated tissue of the embryo.

A. Construction of a retroviral vector

- 30 A replication-defective retroviral vector was prepared from a wild type reticuloendothelial virus, spleen necrosis virus (SNV) as follows. The American Type Culture Collection (ATCC) supplied two spleen necrosis virus (SNV) REVs under accession numbers ATCC 45012 and 45013. ATCC 45013 had a deleted packaging signal.

- 10 -

As is usual in retroviral vectors, the vector included a proviral DNA helper construct having gag, pol and env genes of SNV, but lacking a packaging signal, whereby the native retroviral genes cannot be packaged. It does not possess a long  
05 terminal repeat (LTR) at either end. An SV40 late promoter is provided. This proviral DNA construct is shown in Figure 4.

The other part of the vector was a construct of DNA made from the E. coli. lacZ ( $\beta$ -galactosidase) gene and the packaging signal and env gene of the SNV, as shown in Figure 5. (The sequences from pBR322, a standard commercially available plasmid,  
10 are of no relevance).

The vector DNA (both constructs) was co-infected into a quail cell line "QT 35". A quail cell line was used because ordinary chicken cell lines are infected with avian leukemia virus, which  
15 is another retrovirus, which might introduce an unwanted infectious virus. To assist the infection, a chemical "Lipofectin" (commercially available) was added to the vector DNA.  
B. Preparation of primordial germ cells

Chicken eggs were incubated to stage 14 of development  
20 (roughly 50h). They were swabbed with alcohol and opened in a dish of saline (9g NaCl/litre of water) at 37°C. The embryo was positioned under a hole in a piece of filter paper, cut free from the surrounding tissue and lifted off from the yolk. This left the embryo stretched across a hole in the filter paper. The PGCs  
25 in the germinal crescent to the anterior and sides of the embryo could easily be seen and the tissue containing them was then cut out with fine scissors. Each piece of germinal crescent tissue was placed in a vial with approx. 100 $\mu$ l of Ca- and Mg-free Hanks saline with 0.02% EDTA (ethylene diaminetetraacetic acid) for  $\frac{1}{2}$  to 2h. Alternatively, pieces of germinal crescent tissue were  
30 incubated with 0.0123% trypsin solution (0.25% solution diluted to 1/20) for 5 mins at 37°C, washed, added to trypsin inhibitor for 5 min at 37°C and washed in Hanks saline. Germinal crescent samples were disrupted by drawing them up and down a 1ml syringe  
35 with 21G needle. Samples of the cell suspension were taken for

- 11 -

cell viability tests using erythrosin B stain and for control DNA samples.

C. Transfer of Primordial Germ Cells

The QT 35 cells stuck to the plates of the culture dishes and  
05 the supernatant was sucked off. A fixed volume of supernatant  
was probed with DNA to give an estimate of virion concentration,  
sufficient supernatant was then added to PGCs to give a  
concentration of at least 500 viroids per 150 germinal crescent  
10 tissue cells per  $\mu\text{l}$  of the QT 35 supernatant fluid. Of these  
cells approximately 10 were larger than  $15\mu\text{m}$  diameter and  
considered to be PGCs.

Alternatively a  $20\mu\text{l}$  blood sample was taken from a stage  
15/16 embryo. Such blood samples normally contain circulating  
15 PGCs. The sample was therefore exposed to  $10\mu\text{l}$  of the defective  
retrovirus for  $\frac{1}{2}$  to 1h and a sample of  $10\mu\text{l}$  taken for injection  
into a recipient embryo.

Recipient embryos were incubated to approximately stage 15 of  
development. The shell was swabbed with alcohol and the embryo  
exposed through a hole roughly 1 to  $2\text{ cm}^2$ . A suspension of the  
20 retrovirally infected germinal crescent cells (roughly 100 PGCs)  
in  $10\mu\text{l}$  saline was injected into the heart of the embryo by  
cardiac puncture using a fine glass needle. The shell was sealed  
with tape and returned to the incubator.

At various times (5d, 10d, 18d) embryos were killed. Small  
25 embryos (5d) were simply divided into head and tail regions.  
Larger embryos had the gonads dissected out.

D. Analysis of embryo tissue

After 5d, 10d and 18d embryos were killed for analysis.. At  
5d they were divided into head and tail regions with the  
30 presumptive gonad in the posterior half. At 10d an attempt was  
made to isolate the urogenital region and in later embryos the  
gonad was isolated. Similar tissues were isolated from normal  
embryos.

Tissues of the infected embryos and of various controls were  
35 examined for integration of the retrovirus using PCR (polymerase

- 12 -

chain reaction), R.K. Sarki *et al.*, Science 230, 1350-1354 (1985). Following a wash in phosphate buffered saline, the tissues/cells were resuspended in 40-100 $\mu$ l distilled water, boiled for 15 min and centrifuged for 15 min to remove the debris. Aliquots of the supernatant containing the DNA (10-40 $\mu$ l) were used in 30-cycle PCR reactions (50-100 $\mu$ l) designed to amplify a 519 base pair fragment of DNA at the 5' end of the lacZ gene. The oligonucleotides used in the PCR were 5' GCTATGACCATTGATTACCGA AND 5' CAAATTCAAGACGGCAAACGA which correspond 10 to nucleotides 1281-1300 and 1800-1781 respectively (numbers refer to those in "Nucleotide Sequences" 1985, IRL Press). Each cycle of the PCR consisted of 1 min at 95°C, 1 min at 50°C and 2 min at 74°C. The PCR products were electrophoresed on agarose gels, blotted onto Hybond-N membranes and the lacZ DNA fragment 15 was detected by hybridization to a radiolabelled DNA probe for lacZ.

The results obtained are shown in the Table. No amplification of the 519 base pair lacZ DNA fragment was obtained with DNA isolated from primordial germ cells or from various 20 tissues of the control embryos. DNA isolated from PGCs that had been isolated, infected with the retrovirus and kept overnight in tissue culture was found to direct the amplification of lacZ. DNA from both the anterior and posterior regions of 5d old embryos injected with cells exposed to the retrovirus was found 25 to contain the lacZ. Integration of the retrovirus was also observed in the urogenital tissues of 10d old embryos, and in the isolated gonads from 18 days old embryos.

- 13 -

Table

Results of the PCR-hybridization analysis (+ = amplification detected; - = no amplification detected)

<u>Tissue/Sample</u>	<u>lacZ amplification</u>
1. <u>Controls</u>	
Water	-
Plasmid pCH110 (containing <u>lacZ</u> gene)	+
REV	-
PGCs	-
Vector-infected PGCs	+
Gonads of uninfected hatchlings	-
(b) <u>Embryos injected with Vector-infected PGCs</u>	
5d, anterior half	+
5d, posterior half	+
10d, urogenital region	+
18d, gonad	+

Additionally, the gonads of embryos killed at 18d were examined by Southern blotting (without prior PCR). Samples of 05 DNA were extracted from the gonads, the DNA cut with restriction endonucleases and run on an agarose gel. The gel was then probed for retrovirus and positive hybridisation detected.

These results demonstrate that retroviruses can attach to 10 PGCs, penetrate them and become uncoated. Once in the cell, the viral RNA has to be transcribed into DNA and integrated into the cell DNA if it is to persist in the genome. The present work has shown that primordial germ cells become infected with a suitable defective retrovirus in at least 3 out of 8 experiments. The results indicate (1) that when infected PGCs are transferred to 15 the embryo they become progressively localized in the germinal crescent and (2) that the foreign DNA "signal" becomes enhanced

- 14 -

by cell division during embryogenesis. Since the polymerase chain reaction is specific for DNA the results clearly show that reverse transcription has occurred and that the DNA is persisting in the germ cell line.

- 15 -

CLAIMS

1. A method of introducing foreign nucleic acid into birds, which comprises providing in vitro foreign nucleic acid which it is desired to introduce into the germ line of a bird, introducing  
05 the foreign nucleic acid into explanted primordial germ cells of a bird and then introducing these primordial germ cells into the blood system of a recipient embryo of an incubated egg, at a stage of embryonic development at which introduced primordial germ cells will settle in the germinal ridge.
- 10 2. A method according to Claim 1, which further comprises hatching a chick from the recipient embryonated egg.
3. A method according to Claim 1 or 2 wherein the introduced primordial germ cells are at a germinal crescent or post-germinal crescent developmental stage.
- 15 4. A method according to Claim 1, 2 or 3 wherein the foreign nucleic acid is introduced in vitro into isolated primordial germ cells or into tissue taken from an embryo and which contains primordial germ cells.
- 20 5. A method according to Claim 4 wherein the primordial germ cells containing the foreign nucleic acid are cultured in vitro in the presence of a development inhibitory activity factor.
6. A method according to Claim 4 or 5 wherein the foreign nucleic acid is introduced by transfection with DNA, injection into the primordial germ cells or introduction of a retroviral  
25 vector carrying the foreign nucleic acid.
7. A method according to any preceding claim wherein the ratio of the number of native primordial germ cells of the recipient embryo to the number of introduced primordial germ cells, competing therewith for entry to the germinal ridge, is reduced.
- 30 8. A method according to Claim 7 wherein the native germinal crescent cells of the recipient embryo are destroyed.
9. A method according to any preceding claim wherein the foreign nucleic acid comprises a foreign gene.
10. Birds incorporating in their germ line foreign nucleic acid

- 16 -

introduced by a method claimed in any one of claims 1 to 9 applied to their mother or to any hen which is an ancestor of their parents.

1/3

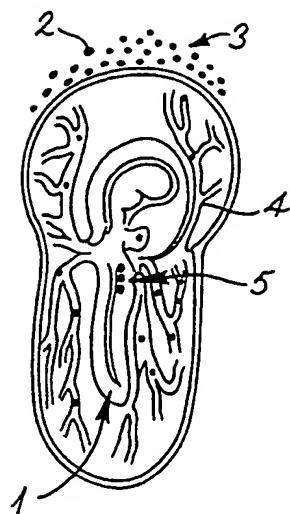


Fig. 1

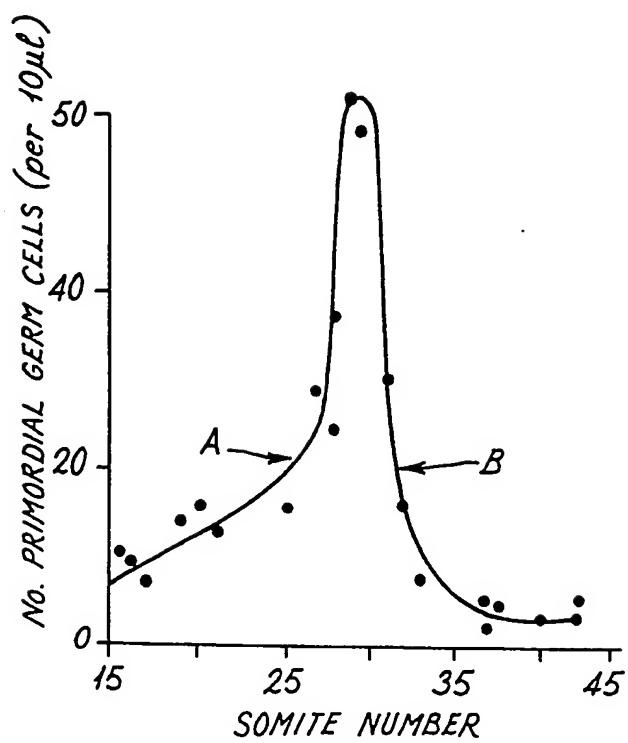


Fig. 2

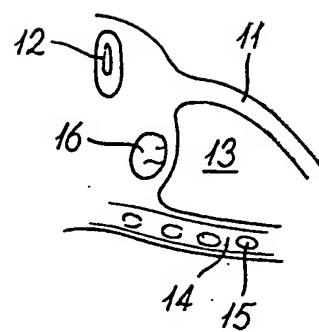


Fig. 3

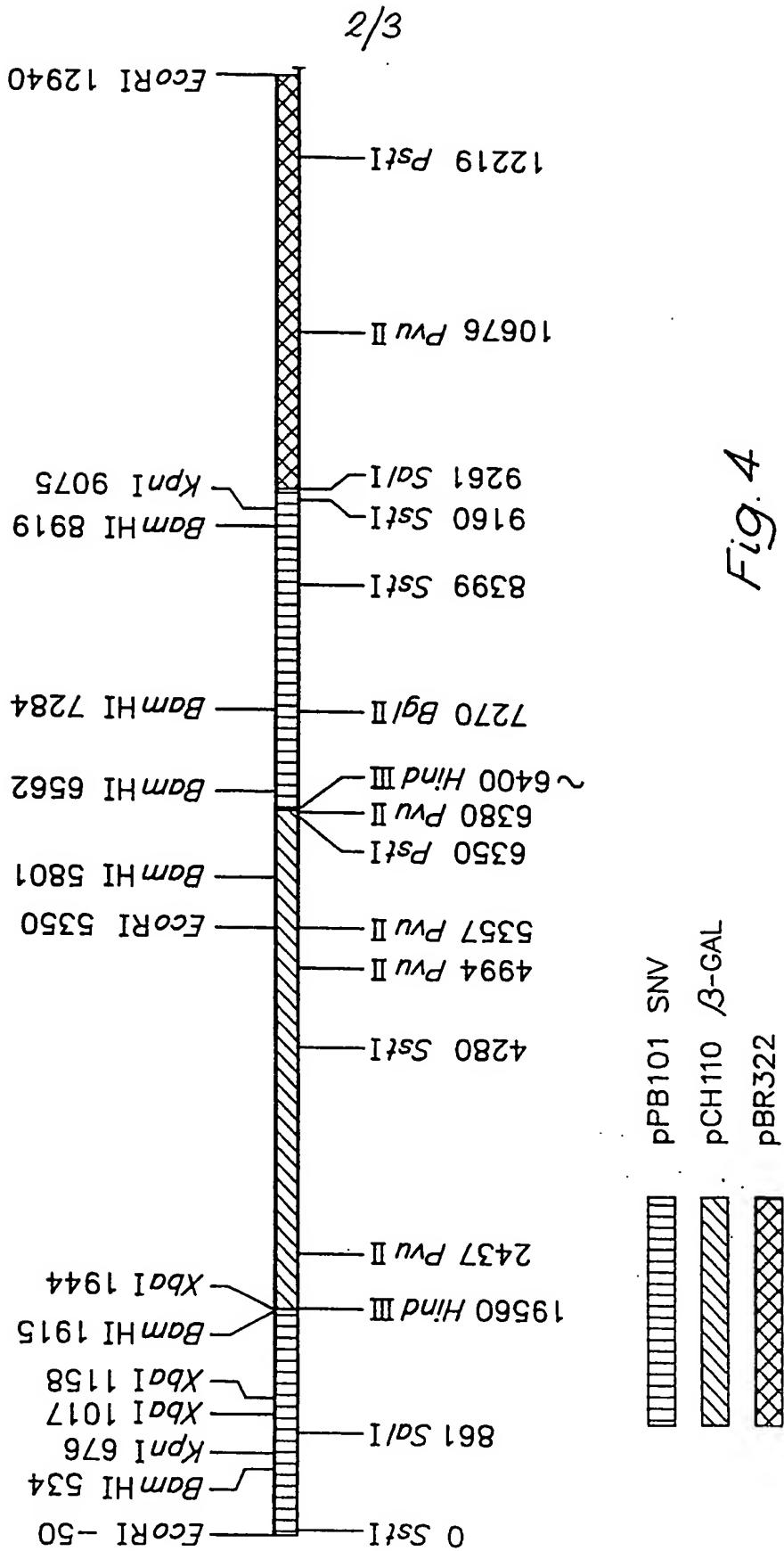


Fig. 4

3/3

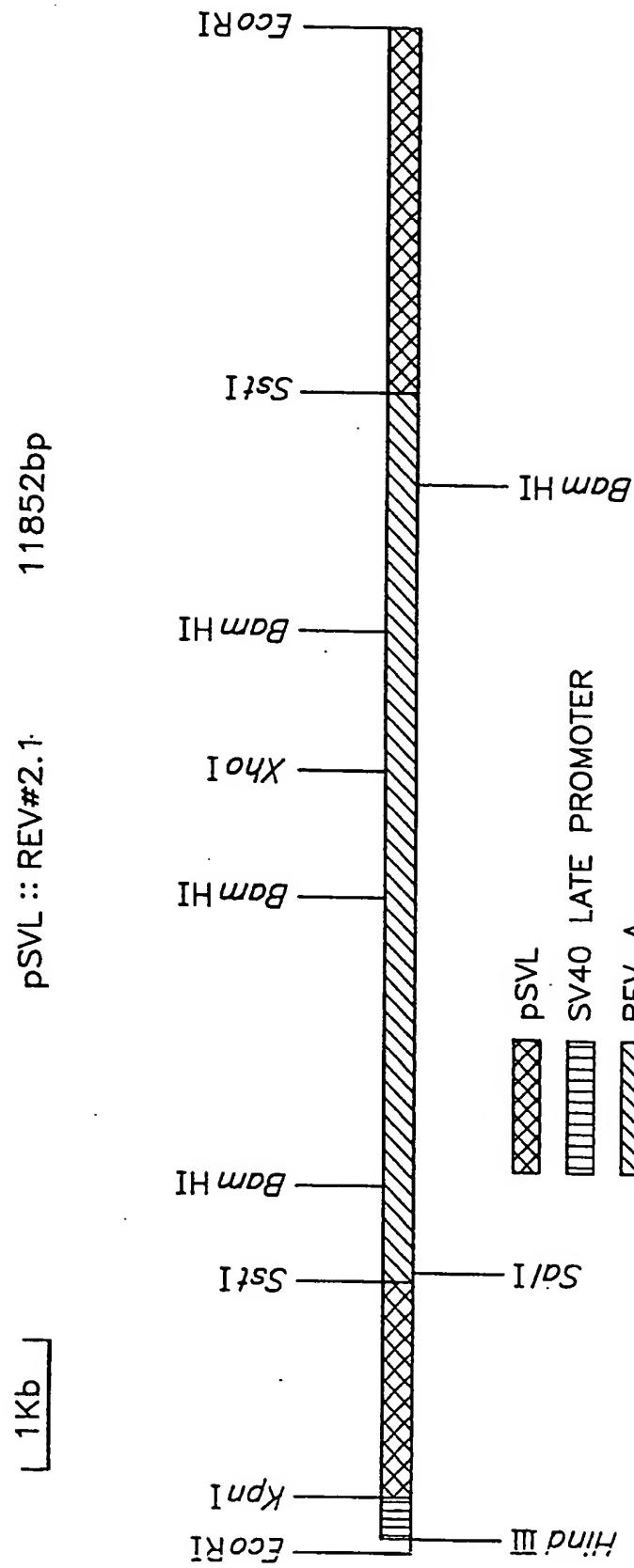


Fig. 5

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 90/00388

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC  
**IPC<sup>5</sup>**: C 12 N 15/00, C 12 N 15/85, A 01 K 67/00

## II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched ?	
	Classification Symbols	
IPC <sup>5</sup>	C 12 N, A 01 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	J. Embryol. exp. Morph., volume 21, no. 3, June 1969, (GB) G. Reynaud: "Transfert de cellules germinales primordiales de dindon à l'embryon de poulet par injection intravasculaire", pages 485-507 see the whole article --	1-5,7-10
Y	These de Doctorat, Université Claude Bernard LYON I, no. 4188, 28 April 1988, F. Chambonnet: "Utilisation de vecteurs rétroviraux dérivés de l'aev (avian erythroblastosis virus) pour le transfert de gènes in vivo chez le poulet", see chapter 2.5.1, and figure 2.3 -- --	1-6,9,10 -- --

\* Special categories of cited documents: 10

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search  
**8th March 1990**

Date of Mailing of this International Search Report

**11.07.90**

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

H. Daniels

H. DANIELS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Trends in Biotechnology, volume 6, no. 5, May 1988, (Cambridge, GB), P.D. Robbins: "Advances in gene transfer and gene therapy", pages 80-83 see page 81, figure 1 --	1
Y	Trends in Genetic, volume 2, January 1986, Elsevier Science Publishers B.V., (Amsterdam, NL), E.J. Robertson: "Pluripotential stem cell lines as a route into the mouse germ line", pages 9-13 see the whole article --	1-6,9,10
A	Science, volume 243, no. 4890, 27 January 1989, (Washington, DC, US), R.A. Bosseman et al.: "Germline transmission of Exogenous genes in the chicken", pages 533-535 see the whole article -----	1,2,6,7,9,10

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**